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Biosafety Markers

Biosafety - HORIZON 2020 EU project

SEMMELWEIS UNIVERSITY, INSTITUTE OF TRANSLATIONAL MEDICINE

TECO*biosciences* GmbH
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Germany

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Outline of the Presentation

- Definitions
- The view of the authorities: guidance documents
- The view of the pharmaceutical industry
- Criteria for the development of NBE and NCE
- Bio(safety)markers for NBE development
- Examples: hematocompatibility testing in nanomedicine
- Examples: immunogenicity – antibodies causing PRCA
- Bio(safety)markers for NCE development
- Example: new liver markers
- Example: testing endocrine disrupting effects

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Definitions

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Biosafety, Biomarker and Biosafety Marker Definition

- The common understanding of *biosafety* is derived from the practical guidance issued by the *World Health Organization* on techniques for use in laboratories. The *WHO Laboratory Biosafety Manual (LBM)* considers biosafety to be "the containment principles, technologies and practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release".
- Biomarkers measure characteristics that are objective and can be evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses.
- (Bio)safety marker are biomarkers that indicate a potential safety concern e.g. in **toxicology**, **immunotoxicology**, **immunogenicity, virological safety environmental health**, etc.

Biomarkers Definition Working Group, NIH Clin Pharmacol Ther 2001;69:89-95

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The View of the Authorities: Guidance Documents

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Toxicology

Guidance for Industry

Drug-Induced Liver Injury: Premarketing Clinical Evaluation

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

July 2009
Drug Safety

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Immuntoxicology

Guidance for Industry

S8 Immunotoxicity Studies for Human Pharmaceuticals

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

April 2006
ICH

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Immuntoxicology

Guidance for Industry

Immunotoxicology Evaluation of Investigational New Drugs

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)

October 2002
Pharmacology and Toxicology

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Immuntoxicology

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

21 February 2013
EMA/CHMP/806058/2009/Rev. 02
Committee for Human Medicinal Products (CHMP)

Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product

Final

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Immunogenicity

Guidance for Industry

Immunogenicity Assessment for Therapeutic Protein Products

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

August 2014
Clinical/Medical

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Environmental Safety

ENV/JM/MONO(2012)22

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 150

GUIDANCE DOCUMENT ON STANDARDISED TEST GUIDELINES FOR EVALUATING CHEMICALS FOR ENDOCRINE DISRUPTION

IOMC INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2012

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The View of the Pharmaceutical Industry

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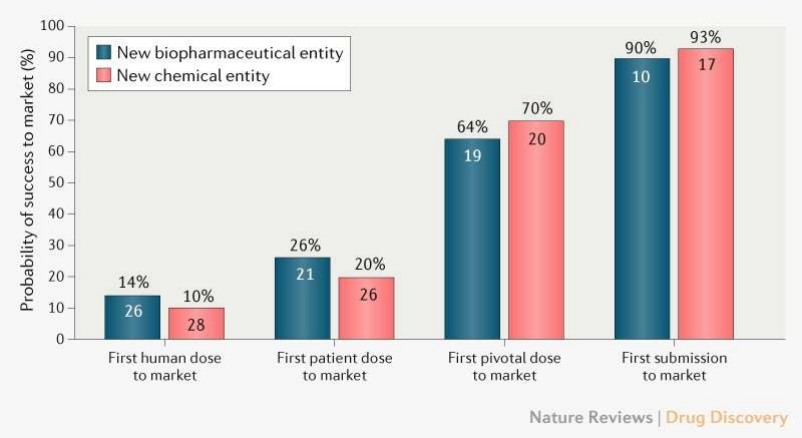
Probability of Success of New Pharmaceutical Entities (NBE or NCE)



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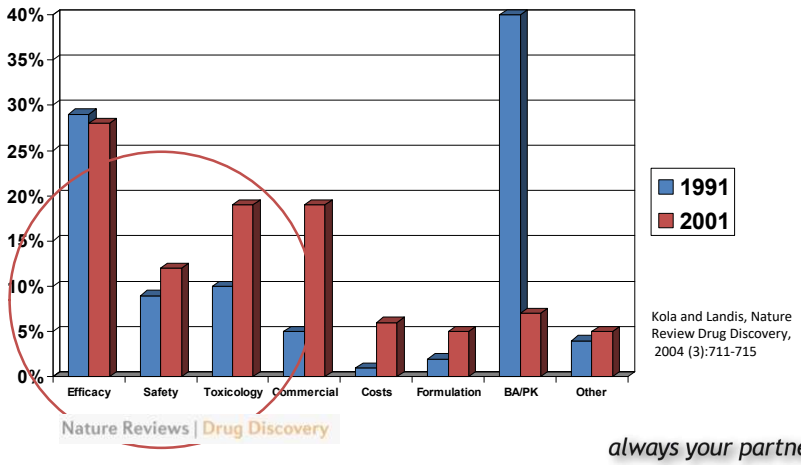
Why Drugs Fail in Development



Kola and Landis, Nature Review Drug Discovery, 2004 (3):711-715

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Driver for Industry to Improve their Success Rate

"We are an industry with a 98% failure rate…..
The only thing we have to do to double our success rate is **to drop our failure rate by 2%."**

Hank McKinnell, Pfizer CEO, at http://www.bio-itworld.com, 2/14/06

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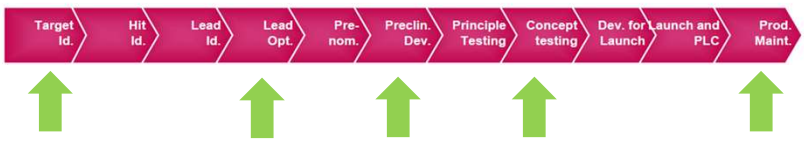
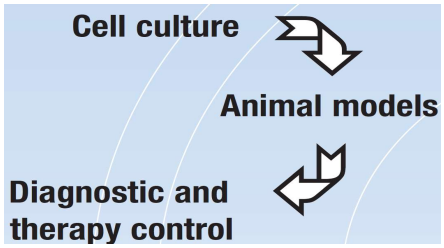
Pharma Needs a Toolbox of Markers that can be used throughout Development





The ultimate goal is to provide a toolbox of qualified (bio)safety & efficacy bio-markers that perform well for drug candidates in preclinical studies (cell culture & animals) and can be used for the same drug candidate to predict and monitor clinical safety in man. Furthermore, to ensure (bio)safety of manufacture.

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Different biosafety markers required for NBE & NCE

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Characteristics of New Biological Entities (NBE) and New Chemical Entities (NCE)

mAb	New Chemical Entity
150,000 dalton	200-500 dalton
Biological production process – heterogeneous (post-translational modifications)	Chemical production process - homogeneous
High species selectivity (affinity / potency)	Generally less selective
Multi-functional – target binding, Fc effector function, FcRn binding	Single target
Toxicity – largely "on target" mediated "exaggerated pharmacology"	Toxicity – often "off target" mediated
Slow clearance; long half-life (days) – infrequent dosing (weekly / monthly) ?	Rapid clearance; short half-life (hours) – frequent dosing (daily)
Target can affect PK behaviour (Target Mediated Drug Disposition)	Mostly linear PK; non-linearity from saturation of metabolic pathways
Drug-Drug Interaction – few examples and mostly PD related	DDI – many examples and metabolic and/or PD related
Immunogenicity sometimes observed	Immunogenicity rarely observed

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Bio(safety) markers used throughout NBE Development

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Key criteria in early NBE development

Discovery: target to lead identification	Preclinical Development	Phase
Manufacturing (MAN) Manufacturability & Quality (adaptation of platform; processes) MAN is quality driven Impurity tests e.g. host cell protein assay	**DMPK/ Immunogenicity** Pharmacokinetics (PK) to study drug exposure Binding antibody (Bab) screening assay & confirmatory assay	**DMPK/ Immunogenicity** PK to study drug exposure BAb screening assay/ confirmatory Assay
Mode of action (MoA) Unique MoA mechanism Proof of biosimilarity New pharmacodynamic (PD) markers	***For high-risk molecules:*** Neutralizing antibody (NAb) test Competitive ligand binding assay for antagonistic drugs & cell-based assay for agonistic drugs	***For high-risk molecules:*** Neutralizing antibody (NAb) tests
Safety De-immunization/humanization	New PD markers	**Toxicology/Safety** Injury markers (liver / kidney) to study potential impact of single and chronic treatment in patients
Confirmation of de-immunization by *in silico*/*in vitro* methods For new molecule classes redundancy assessment in transgenic/surrogate animals	**Toxicology (Tox)/Safety** Classical tox testing	

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Key criteria in late NBE development

Phase II	Phase III	Post Market
DMPK and MoA PK/PD Potential identification of inclusion/exclusion criteria (prognostic and predictive markers)	**Manufacturing (MAN)** Improving process for market registration (quality/costs) Potential new formulations	**Manufacturing (MAN)** Quality and environmental markers
Toxicology/ Safety BAb Screening assay Confirmatory assay NAb assay	**DMPK and MoA** PK/PD Application of potential prognostic and predictive markers	**DMPK and MoA** Application of potential prognostic and predictive markers
Injury markers (liver / kidney) in case of potential side effects observed in phase I	**Toxicology/ Safety** BAb Screening assay Confirmatory assay NAb assay Sophisticated injury markers (liver / kidney) to monitor chronic treatment in large patient groups	**Toxicology/ Safety** Single biomarkers (immunogenicity/ liver / kidney) if shown to indicate risks for long term treatment
Hypersensitivity testing on demand (prick test, RAST, PK/PD, CARPA)		

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EXAMPLE

Pseudo-allergies and Infusion Reactions upon Treatment with Antibodies and Innovative Formulations (Nanomedicine)

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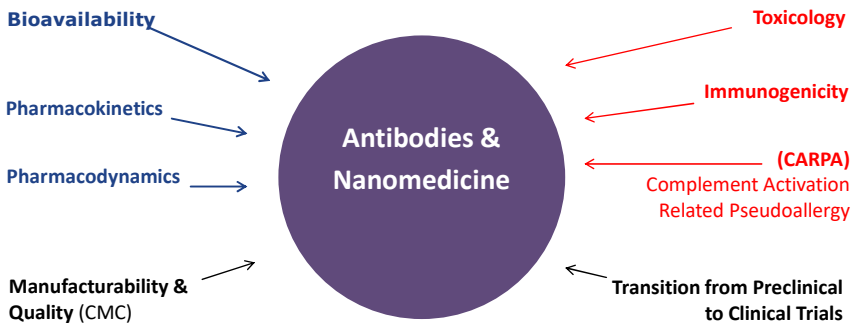
Critical Factors to Consider for Successful Development of Antibodies & Nanomedicine



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Types of Hypersensitivity Reactions

Type	Alternative Name	Examples	Mediators
I	Allergy (immediate)	• Anaphylactic reactions • Asthma • Allergic rhinitis • Angiodema • Food allergies	IgE
II	Cytotoxic (antibody dependent)	• Autoimmune anemias e.g. thrombocytopenia • Erythroblastosis fetalis	IgG, IgM
III	Immune complex disease	• Serum sickness • Reactive arthritis • Arthus reaction	Aggregation of antigens IgG, IgM and complement proteins
IV	Delayed hypersensitivity	• Contact dermatitis • Chronic asthma, rhinitis • Chronic transplant rejection	T-cells, monocytes, macrophages
Pseudo-allergies	Complement activation-related pseudoallergy (CARPA)	• Infusion reactions	Anaphylatoxin-triggered

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Drugs and Agents Causing Pseudoallergies (Examples)

- **All marketed liposomal drugs**
  Doxil (Caelyx), Ambisome, Myocet, Abelcet, Amphotec, Amphocyl, DaunoXome, Visudyne
- **Some micellar anticancer drugs**
  Fasturec, Elitec, Taxol, Cyclosporine, Vumon, Etoposide, Taxotere
- **Some PEGylated proteins**
  Adagen, Neulasta, Oncaspar, Pegaspargase
- **Most radio and ultrasound contrast agents**
  Diatrizoate, Iodixanol, Iohexol, Iopamidol, Iopromide, Iothalamate, Ioversol, Ioxaglate, Ioxilan, SonoVue, Magnevist
- **Most enzymes**
  Avonex, Actimmune, Abbokinase, Aldurazyme, Activase, Zevalin, Neupogen, Neulasta, Fasturtec, Plenaxis

Szebeni et al. J. Eur. J. Nanomedicine, 4 (1), 33-53

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Overview Complement Marker

Activation products - all pathways
C3a, iC3b, C5a, SC5b-9
C3d
Activation products - classical pathway
C1-inhibitor
C4a, C4d
Activation products - alternative pathway
C3b, Bb,
Ba, Bb
Activation terminal sequence
C5a, SC5b-9
Regulators
Factor H and Factor I protein
Factor H and Factor I function
Factor P - Properdin

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EXAMPLE

Immunogenicity of Protein Drug Product (DP): Influence of Processing and Storage Conditions on Immunogenicty

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Immunogenicity of Protein Drug Product : Influence of Processing and Storage Conditions (I)

- Influence of processing conditions
  - Temperature, freeze-thawing
  - Shear stress (pumping-induced aggregation)
  - Exposure to liquid-solid interface (without surfactant)
  - Light exposure
- Influence of storage and shipping conditions
  - Primary packaging (vial or PFS with silicone coating),
  - Temperature,
  - Stirring, shaking (shipping)
  - Light exposure

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Immunogenicity of Protein Drug Product : Influence of Processing and Storage Conditions (II)

- All proteins absorb light, especially by tryptophan (Trp) residues, albeit to different degrees:
  - Photo-induced degradation can occur at many points from manufacturing to storage and delivery
  - Photo-induced degradation can affect shelf-life stability of drug product: both EMA and FDA require photostability studies for protein-based drug approval
- Mechanisms:
  - Irradiation causes disruption of secondary structure (photolysis) and loss of secondary structure correlates with formation of aggregates
  - Proteins with multiple disulfide bonds are more susceptible to light-induced aggregation
    - Example: Antibodies: disulfide bond cleavage and reactions with oxygen or with other amino-acids (cross-linking)

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Immunogenicity of Protein Drug Product : Influence of Processing and Storage Conditions (III)

- Influence of foreign particles leaching from pumps/container/closure:
  - Foreign particles behave as process-derived impurities in the DP and may trigger immune responses
  - Foreign particles may induce nucleation of protein (hetero-)aggregates
- Examples of foreign particles generated during processing:
  - Stainless steel and other particles from pumps (Carpenter et al., 2006)
  - Glass (nano)particles generated during heating of containers for de-pyrogenation (Chi et al., J. Pharm. Sci. 2005)
  - Free silicone oil or particles generated during container siliconization
  - Rubber and/or silicone leachable and/or particles from stoppers

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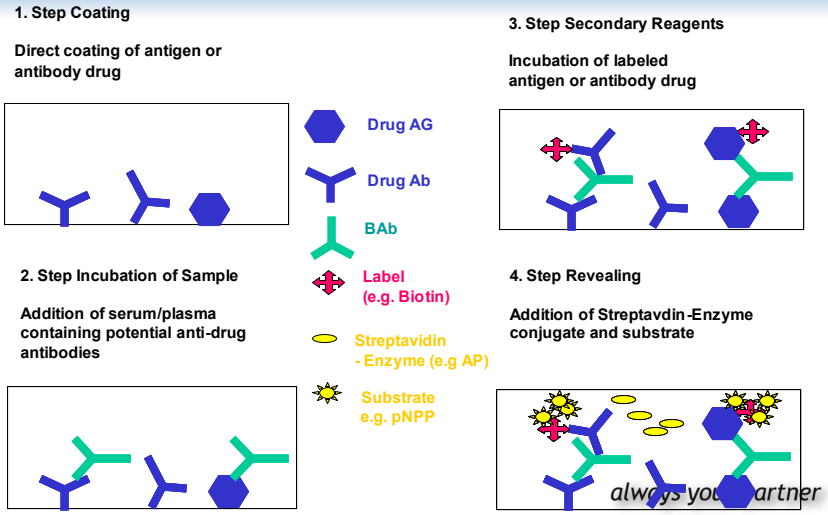
How to detect Immungenicity: ELISA Bridging Format Principle as Biosafety Assay



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Bridging ELISA Pro's and Con's

Pro's

- Rapid
- Can be automated
- Easy to handle
- High specificity due to monovalent binding, good reproducibility, accuracy, etc.
- High throughput
- Same reagents for samples and controls
- Species independent

Con's

- Tend to show matrix effects
- Lower sensitivity than ELISA direct format
- Some formats e.g. those involving immobilization of antigen may alter/mask antigen conformation
- May fail to detect rapidly dissociating antibodies (low affinity antibodies)
- Conjugation chemistry of the antigen may have an impact on molecular structure of the drug

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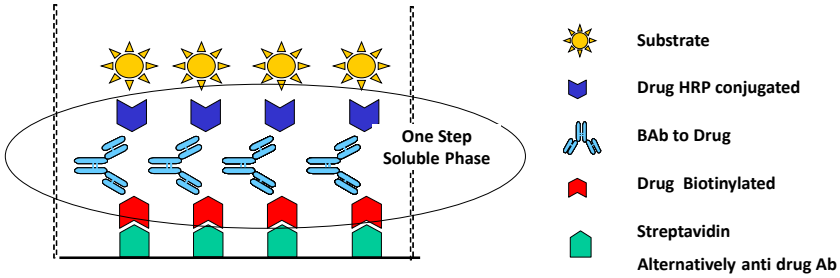
How to detect Immungenicity: ELISA Indirect Format Principle as Biosafety Assay

One step bridging ELISA in wash out samples



- Use of Streptavidin pre-coated plates to minimize coating variability and to reduce assay background
- One step incubation of biotinylated and HRP conjugated drug with serum to reduce turn around time (1-2 hours)
- Reduced washing steps allow the detection of low affinity antibodies
- Drug kept in oriented position which increases sensitivity

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ELISA Indirect Format Pro's and Con's

Pro's

- Very rapid (1-2 hours)
- Can be automated
- High throughput
- Easy to handle
- Good sensitivity, high specificity, accuracy, reproducibility, etc.
- Same reagents for samples and controls
- Species independent
- May detect low affinity antibodies (reduced washing steps)

Con's

- Pre-coated plates are expensive
- Conjugation chemistry of the antigen may have an impact on molecular structure of the drug
- In case of pre-coating with anti-drug Ab investigations have to be carried out to ensure that binding of potential BAb to drug is not inhibited

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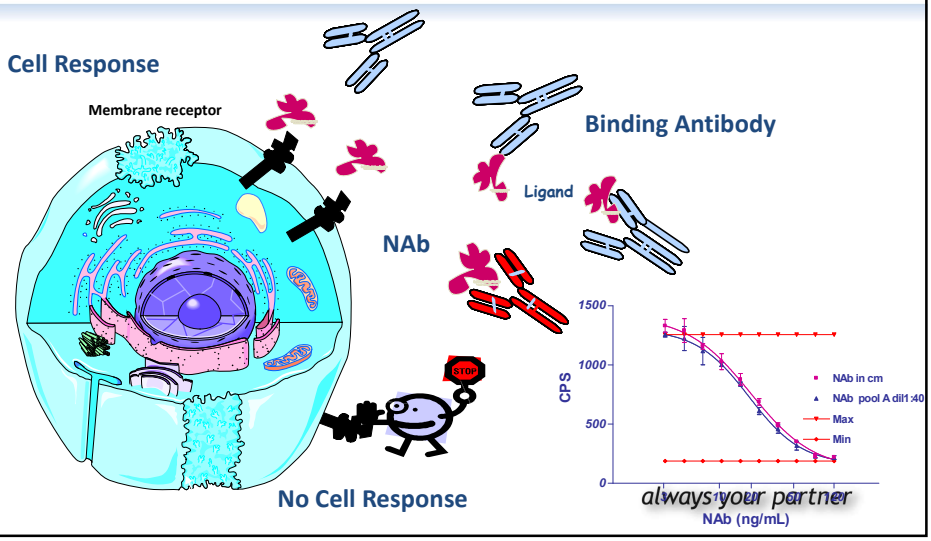
Cell Based Assays to Detect Neutralizing Anibodies as Bioasafety Assay



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Case Study: EPO-a EPREX® Immunogenicity Can Cause Severe Side Effects

The New England Journal of Medicine

Volume 346:469-475 February 14, 2002 Number 7

Pure Red-Cell Aplasia and Antierythropoietin Antibodies in Patients Treated with Recombinant Erythropoietin

Nicole Casadevall, M.D., Joelle Nataf, M.D., Béatrice Viron, M.D., Amir Kolta, M.D., Jean-Jacques Kiladjian, M.D., Philippe Martin-Dupont, M.D., Patrick Michaud, M.D., Thomas Papo, M.D., Valérie Ugo, M.D., Irène Teyssandier, B.S. Bruno Varet, M.D., and Patrick Mayeux, Ph.D.

Dept of Haematology, Hôtel-Dieu, Paris, France

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Epogen®/Procrit® (US)	Eprex® (pre 1998)	Eprex® (post 1998)	NeoRecormon® (launch in1990)
hSA	hSA (Vials)	Polysorbate 80	Polysorbate 20
		Glycine	Glycine
		(PFS/Uncoated stoppers)	Complex of 5 amino acids
	Appearance of antibody-positive PRCA cases in Eprex®-treated patients, starting in 1998		Calcium chloride
			Urea

PRCA= pure red cell aplasia

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Case Study: Immunogenicity of EPO-a EPREX® Conclusions & Lessons (I)

RESEARCH

Recognition and Identification of UV-absorbing Leachables in EPREX® Pre-filled Syringes: An Unexpected Occurrence at a Formulation–Component Interface

JAMES PANG,[1] TIM BLANC,[2] JOHN BROWN,[1] STEVEN LABRENZ,[3] ANNABELLE VILLALOBOS,[4] ANNELI DEPAOLIS,[3] SRINIVAS GUNTURI,[3] STEVE GROSSMAN,[5] PETER LISI,[1] GEORGE A. HEAVNER[6]

[1]Global Analytical Services, Global Biologics Supply Chain, LLC, 1000 Route 202 South, Raritan, NJ 08869; [2]Johnson and Johnson Pharmaceutical Research and Development, 2000 Route 202 South, Raritan, NJ 08807; [3]Process Technology, Global Biologics Supply Chain, LLC, 200 Great Valley Parkway, Malvern, PA 19355; [4]Global Technical Services, Global Biologics Supply Chain, LLC, 1000 Route 202 South, Raritan, NJ 08869; [5]Quality Control, PSGA, 1000 Route 202 South, Raritan, NJ 08869; [6]Centocor Research and Development, 145 King of Prussia Road, Radnor, PA 19087

PDA Journal of Pharmaceutical Science and Technology

Vol. 61, No. 6, November-December 2007

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- Immunogenicity models and clinical data analysis suggested that leachables are at the origin of the PRCA:
  - Adjuvant-like effect of the leachables was confirmed in mouse models
  - Generation of anti-EPO Abs in mice co-injected with leachables/rHuEPO
- Uncoated stoppers were replaced by FluroTec-coated stoppers, which brought back the incidence of PRCA to baseline
- Lessons:
  - Too many changes in a final dosage form at the same time without appropriate change control may have unexpected, disastrous effects! Complete, extensive risk assessment has to be performed.
  - Compatibility of DP components (formulation/container) must be carefully monitored
  - Biopharma companies have carried out an outstanding piece of work to understand the origin of the issue and bring corrective actions.

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Bio(safety) markers used throughout NCE Development

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Key criteria in early NCE development

Discovery: target to lead identification	Principal Development	Phase !
Manufacturing (MAN) Manufacturability (e.g. solubility, stability) MAN cost driven	**DMPK** Pharmacokinetic assay (PK) to study drug exposure Bioavailability (i.v. vs oral administration) Identification of metabolites	**Manufacturing (MAN)** Improving process to reduce Impurities identified in preclinical development
Mode of action (MoA) Unique MoA mechanism Replacement of NBE using same target	**Mode of action (MoA)** Identification of metabolites and their activity including toxicity Identification of biomarkers to study pharmacodynamics (PD)	**DMPK and MoA** PK/PD
Toxicology Screening of active principle (API) By in vitro and in vivo (LD50) Testing in single dose studies	**Toxicology/Safety** Sophisticated markers (liver / kidney) to study potential impact of chronic treatment in vivo	**Toxicology/Safety** Liver and kidney markers to study potential impact of single and chronic treatment in patients

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Key criteria in late NCE development

Phase II	Phase III	Post Market
DMPK and MoA PK/PD Potential development of assays to study metabolite exposure Potential identification of inclusion/exclusion criteria (prognostic and predictive markers)	**Manufacturing (MAN)** Improving process for market Registration (quality/costs) Environmental markers	**Manufacturing (MAN)** Quality and Environmental markers
Toxicology/ Safety Sophisticated markers (liver / kidney) to study potential impact of chronic treatment in patients	**DMPK and MoA** PK/PD Application of potential prognostic and predictive markers	**DMPK and MoA** Application of potential prognostic and predictive markers
	Toxicology/ Safety Injury markers (liver / kidney) to monitor chronic treatment in large patient groups	**Toxicology/ Safety** Single biomarkers (liver / kidney) if shown to indicate risks for long term treatment

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EXAMPLE
Novel Liver & Kidney Injury Markers

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Drug-induced Liver Injury (DILI)

- Drug-induced liver injury (DILI) now ranks as the leading cause of liver failure and transplantation.
- USA C-Path's Predictive Safety Testing Consortium (PSTC; partnership FDA) + EU Innovative Medicines Initiative (IMI) SAFE-T consortium:
  - *Current standards (Aspartase Transaminase AST, Alanine Transaminase, ALT, Bilirubin) are not specific and do not predict who will recover or develop severe liver disease.*
  - *Species differences in drug toxicity in preclinical safety tests, lack of sensitive translational biomarkers cause failures in predicting human drug toxicity.*
  - *Changes in drug discovery practices and the implementation of specific and sensitive Safety Biomarkers are expected to decrease considerably these drug development failures.*

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Overview Liver Injury Marker

Liver Biomarker	Tissue Specificity	Indicative for	Species		Cell culture
	LIVER (SERUM)		HUMAN	ANIMAL	
α GST *(Alpha Glutathione S-Transferase)*	Hepatocytes	Necrosis	√		√ *(2D & 3D)*
π GST *(Pi Glutathione S-Transferase)*	Bile duct	Necrosis	√	-	√
ccK18 *(Caspase - cleaved Keratin 18: M30 Elisa*	Hepatocytes	Apoptosis	√	Monkey Bovine	√ *(2D & 3D)*
K18 *(cleaved and uncleaved Keratin 18: M65 Elisa)*	Hepatocytes	Necrosis and Apoptosis	√	Monkey	√ *(2D & 3D)*

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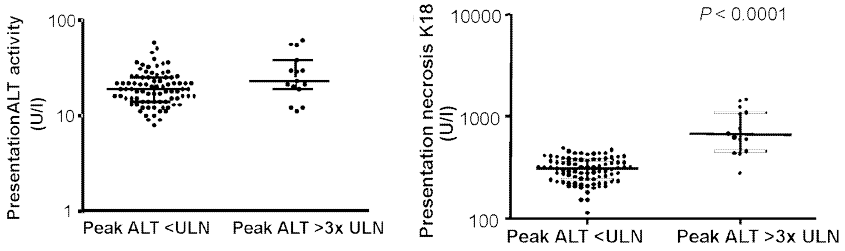
Paracetamol – Liver Toxicity

Prediction of severe liver injury



Comparison cK18 and ALT levels in subjects whom have overdosed on paracetamol and whom are at risk of developing severe liver injury, defined by an ALT level > 3x ULN. Only cK18 predicted the injury.

Antoine et al Hepatology 2013

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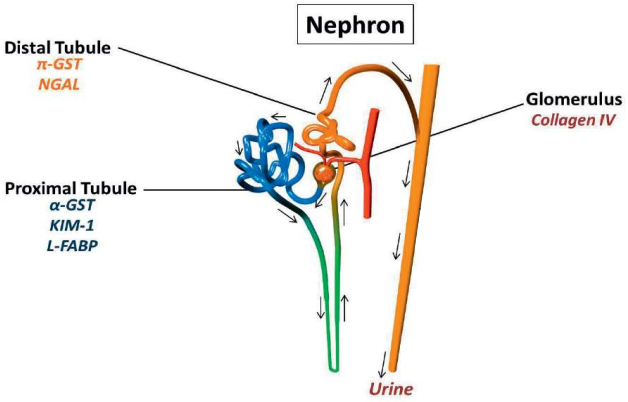
Kidney Injury Biomarkers



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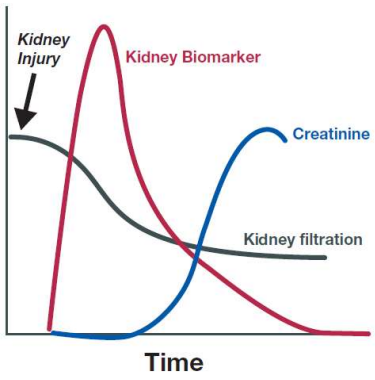
Response Time Kidney Markers

50 % of kidney function is lost before Acute Kidney Injury (AKI) is detected by elevated serum creatinine levels.

Novel kidney injury urinary biomarkers can detect AKI as early as 4-6 hours following the initial kidney injury (creatinine:

24-48 hours). Use of these kidney bio-markers allows early and site-specific prediction of kidney injury in



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Overview Kidney Injury Marker

Kidney Biomarker	Tissue Specificity	Indicative for	Response time following injury*	Species		Cell culture
	KIDNEY (URINE)			HUMAN	ANIMAL	
α GST *(Alpha Glutathione S-Transferase)*	Proximal tubule	Necrosis	Within 4-6 hours	√		√ *(2D & 3D)*
π GST *(Pi Glutathione S-Transferase)*	Distal tubule	Necrosis	Within 6 hours	√	-	√
KIM-1 *(Kidney Injury Molecule-1)*	Proximal tubule	Regeneration after injury	Within 12-24 hours	√	Rat	
L-FABP *(Liver Type Fatty Acid Binding Protein)*	Proximal tubule	Necrosis	Within 4-6 hours	√		
NGAL *(Neutrophil Gelatinase Associated Lipocalin)*	Distal tubule	Regeneration after injury	Within 4-6 hours	√		
Collagen IV	Glomerulus	Glomerular damage	Chronic deposition	√		

** response times displayed are indications.*

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Success Requires Strong Collaboration

SAFE-T

Innovative Medicines Initiative

International SAE Consortium

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

FDA

CRITICAL PATH INSTITUTE
Improving the Path for Innovative Therapies

THE biomarkers CONSORTIUM

FOUNDATION FOR THE National Institutes of Health

Pmda Pharmaceuticals and Medical Devices Agency, Japan

ILSI HEALTH AND ENVIRONMENTAL SCIENCES INSTITUTE

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EXAMPLE
Novel Test to Study
Endocrine Disrupting Effects

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Endocrine Disruptors - Definition

- Endocrine disruptors are chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife.
- A wide range of substances, both natural and man-made, are thought to cause endocrine disruption, including pharmaceuticals, dioxin and dioxin-like compounds, polychlorinated biphenyls, DDT and other pesticides, and plasticizers such as bisphenol A.

http://www.niehs.nih.gov/health/topics/agents/endocrine/

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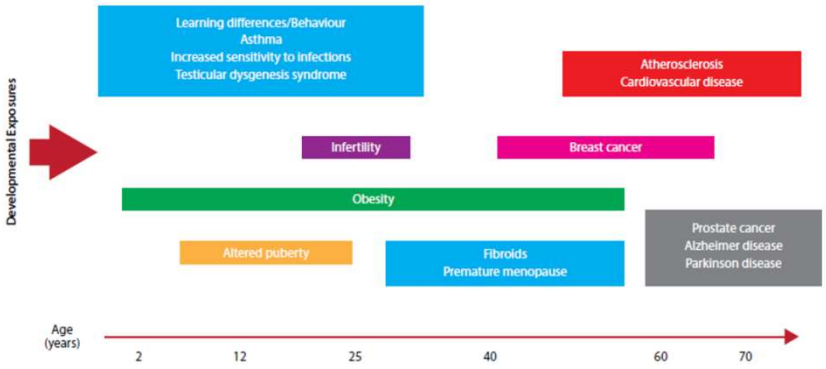
Overview: Endocrine Disruptors



WHO 2012 Endocrine Disrupting Chemicals

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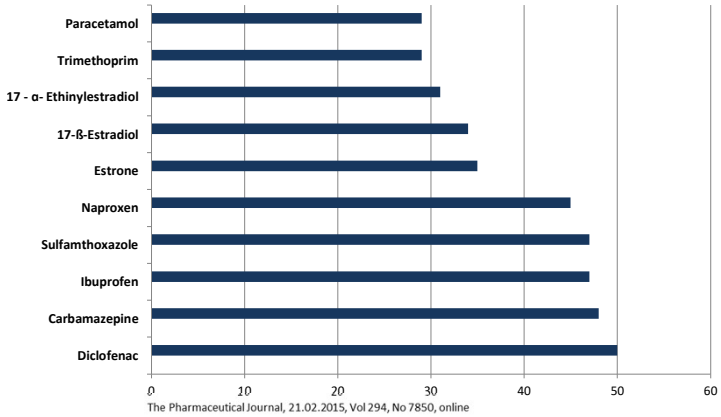
The Top Ten Endocrine Disrupting Drugs Found in the Aquatic Environment



The Pharmaceutical Journal, 21.02.2015, Vol 294, No 7850, online

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Adverse Effects of Drugs on Non-Target Organisms

Examples

Active substance	Use	Non-target organism	Effect	Study	Reference
Ethinylestradiol	Human Pharmaceutical Hormon	Fathead Minnow	Population collapse due to feminization of male fish	Field trial	Kidd KA, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW (2007): Collapse of A fish Population after Exposure to synthetic estrogen. Proceedings of the National Academy of Sciences of the United States of America 104 (21): 8897-8901
Ethinylestradiol	Human Pharmaceutical Hormon	Fathead Minnow	Inhibition of reproduction	Laboratory	Länge R, Hutchinson TH, Croudace CP, Siegmund F, Schweinfurth H, Hampe P,Panter GH, Sumpter JP (2001): Effects of the synthetic estrogen 17 alpha-ethinylestradiol on the life-cycle of the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry. 20(6): 1216
Ethinylestradiol	Human Pharmaceutical Hormon	Carp	Female characteristics in male	Environmental effect	Petrovic M, Solé M, López de Alda M, Barceló D (2002): Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: Integration of chemical analysis and biological effects on feral carp.Environmental Toxicology and Chemistry. 21(10): 2146–2156
Ethinylestradiol	Human Pharmaceutical Hormon	Zebra fish	Change of male sexual organs	Laboratory	Nash JP, Kime DE, Van der Ven LT, Wester PW, Brion F, Maack G, Stahlschmidt-Allner P, Tyler CR (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ Health Perspect 112(17): 1725-1733
Propanolol	Human Pharmaceutical Beta blocker	Medaka	Inhibition of growth	Laboratory	Huggett DB, Brooks BW, Peterson B, Foran CM, Schlenk D (2002): Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms. Arch. Environ.Contam. Toxicol. 43 (2002):229–23
Propanolol	Human Pharmaceutical Beta Blocker	Hyalella azteca	Disturbance of propagation	Laboratory	Huggett DB, Brooks BW, Peterson B, Foran CM, Schlenk D (2002): Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms. Arch. Environ.Contam. Toxicol. 43 (2002):229–23

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Ways of pharmaceuticals get into environment

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Pharmaceutical products with endocrine disrupting activities of increasing governmental and public concern

- EU-Guidance 2001/83/EG; 2004/27/EG demand testing for ecotoxicological effects
- Different activities to increase regulatory requirements on EU level
- Regulations by German Arzneimittelgesetz (AMG)

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EU Human Pharmaceuticals Regulation (EC 726/2004) and OECD Guidelines for testing

	OECD TG 210 (1992)	OECD TG 229 (2009)	OECD TG 230 (2009)*	OECD TG 234 (2011)	OECD TG 305 (1996)
Title Legislation	Fish early life-stage toxicity test	Fish short-term reproduction assay (21 days) (Fathead Minnow)	21-Day fish screening assay (Fathead Minnow, Medaka, Zebrafish)	Fish sexual development test (60 days)	Bioconcentration: Flow-through fish test
EU Human Pharmaceuticals (Regulation EC 726/2004)	Base set requirement for Phase II TierA	Conditional requirement	Conditional requirement	Probably on an ad hoc basis, if concern for endocrine disruption	Required for PBT screening, of log Kow is ≥4.5 and in Tier B if log is ≥3
	Criteria	Daily measurement of egg products Vitellogenin Level Secondary sexual characteristics Histology of gonads	Vitellogenin Level (Fathead Minnow, Medaka, Zebrafish) Secondary sexual characteristics (Fathead Minnow, Medaka)	Vitellogenin level Phenotypic and genotypic sex ratio	

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Testing for Endocrine Disruptors with Estrogenic Activities - Fish Vitellogenin

- Vitellogenin (vtg) is a precursor for the egg yolk proteins lipovitellin and phosvitin - its synthesis normally occurs only in sexually active females and therefore Vitellogenin is considered as a „female-specific protein"

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Testing for Endocrine Disruptors with Estrogenic Activities - Fish Vitellogenin Cont'd

- The presence of vtg in blood and liver of male and immature fishes is widely used as an indicator of endocrine disruption, since the production is dependent on the presence of estrogen effective substances which should not occur in those organisms under normal circumstances. Therefore vtg is regarded as a reliable biomarker of exposure to estrogenic pollutants in both for in vitro (e.g., hepatocyte cultures) and in vivo studies.

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Classical Vitellogenin Testing

- Specific ELISA mainly for Carp, Zebrafish, Fathead Minnow, Medaka and Rainbow Trout
- Sample Type: Blood, Homogenate
  - Invasive
  - Destructive
  - Anesthesia required
- Directed for OECD testing in laboratory
- No routine usage for ecotoxicological testing and environmental monitoring

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New Vitellogenin Testing

- New Generation of ELISA detecting a broad range of fish species (27 fish species including marine species)
- Classical sample types: Blood, Homogenate
- New sample type: Epidermal mucus
  - Easy swabbing method
  - Non-invasive/Non-destructive
  - Preserves the integrity of fish
  - Repeated testing possible
- Directed for OECD testing in laboratory
- Directed for ecotoxicological testing and environmental monitoring

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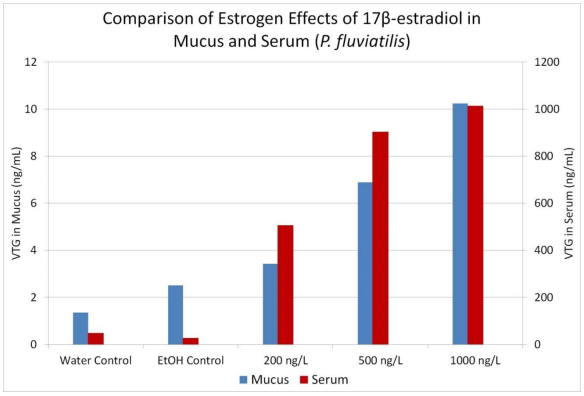
Dose-depending induction of vtg in serum and mucosa by exposure of P. fluviatilis to 17-ß estradiol after six days.



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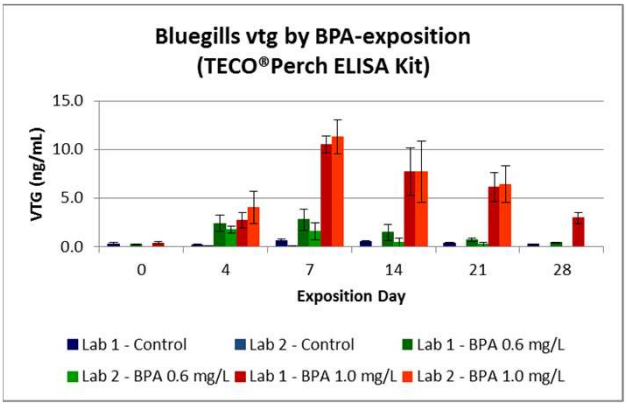
Repeated Vitellogenin measurement demonstrate dynamic effects of endocrine disruptors in fish



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Advantages of new Vitellogenin Testing

- Vitellogenin measurement in mucus gives similar results compared to blood and homogenate
- Repeated testing in mucus allows studying endocrine disruptive effects of pharmaceutical compounds in fish
- Detection of mucus vitellogenin in a broad range of fish species allows extensive ecological testing and environ-mental monitoring

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Questions

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